Mechanisms involved in the resistance of *Enterococcus faecalis* to calcium hydroxide

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Abstract

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Aim This study sought to clarify the mechanisms that enable *E. faecalis* to survive the high pH of calcium hydroxide.

Methodology *E. faecalis* strain JH2-2 was exposed to sublethal concentrations of calcium hydroxide, with and without various pretreatments. Blocking agents were added to determine the role of stress-induced protein synthesis and the cell wall-associated proton pump.

Results *E. faecalis* was resistant to calcium hydroxide at a pH of 11.1, but not pH 11.5. Pre-treatment with calcium hydroxide pH 10.3 induced no tolerance to further exposure at pH 11.5. No difference in cell survival was observed when protein synthesis was blocked during stress induction, however, addition of a proton pump inhibitor resulted in a dramatic reduction of cell viability of *E. faecalis* in calcium hydroxide.

Conclusions Survival of *E. faecalis* in calcium hydroxide appears to be unrelated to stress induced protein synthesis, but a functioning proton pump is critical for survival of *E. faecalis* at high pH.

Keywords: *E. faecalis*, proton pump, sodium hypochlorite, stress response.

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Introduction

Only a few studies have investigated the aetiology of failures in which the endodontic therapy has been well performed. These studies have revealed five factors that may contribute to a persistent periapical radiolucency after treatment. The factors are: intraradicular infection (Nair *et al.* 1990a); extraradicular infection by bacteria of the species *Actinomyces israelii* and *Propionibacterium propionicum* (Nair & Schroeder 1984, Sjögren *et al.* 1988); foreign body reaction (Koppang *et al.* 1989, Nair *et al.* 1990b); cysts, especially those containing cholesterol crystals (Nair *et al.* 1993); and fibrous scar tissue healing following conventional treatment (Nair *et al.* 1999). Of all these factors, it is generally believed that the major cause of root canal treatment failure is the persistence of microorganisms in the apical part of root-filled teeth.

Although information on the type of microorganisms that persist in the root-filled canal is limited, several studies have reported that root-filled teeth with persisting periapical lesions usually harbour one or a few bacterial species and that Gram-positive bacteria dominate the bacterial flora (Möller 1966, Molander *et al.* 1998, Sundqvist *et al.* 1998, Noda *et al.* 2000, Hancock *et al.* 2001). The species *Enterococcus faecalis* has been recovered in a high proportion of endodontic failures, approximately one third of the canals of root-filled teeth with persistent periapical lesions (Möller 1966, Molander *et al.* 1998, Sundqvist *et al.* 1998, Hancock *et al.* 2001).

The common recovery of *E. faecalis* from canals where the treatment has failed suggests it is an opportunistic pathogen whose persistence in the root canal presents a significant therapeutic problem. When *E. faecalis* has been identified in cases of failed endodontic treatment, it has proven to be difficult to eradicate during retreatment (Sundqvist *et al.* 1998), and the failure rate of retreatment is higher when *E. faecalis* has been recovered from the root canal at the time of root filling (Sundqvist *et al.* 1998). Once established in the root canal, *E. faecalis* faces

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several challenges for survival, including an ability to withstand antimicrobial agents used during treatment and endure potential starvation in the cleaned and obturated canal. *E. faecalis* appears to be highly resistant to the medicaments used during treatment and is known to resist the antibacterial effect of dressing with calcium hydroxide (Byström *et al.* 1985, Haapasalo & Ørstavik 1987, Safavi *et al.* 1990, Ørstavik & Haapasalo 1990, Sato *et al.* 1993, Weiger *et al.* 1995, Siqueira & de Uzeda 1996, Tanriverdi *et al.* 1997, Waltimo *et al.* 1999). Interestingly, resistance to killing by calcium hydroxide is shared with the few other microorganisms that have been associated with endodontic failure, such as Candida species (Nair *et al.* 1990a, Waltimo *et al.* 1997, Waltimo *et al.* 1999) and *Actinomyces radicidentis* (Kalfas *et al.* 2001).

Although *E. faecalis* has been reported to withstand the high pH of calcium hydroxide, relatively little is known about the survival mechanisms of *E. faecalis* that enable it to tolerate exposure to calcium hydroxide. In general, when bacteria face an adverse or potentially lethal challenge, a stress response is mounted that allows them to endure the threat, survive and recover (Parsell & Lindquist 1993, Welch 1993). This response has been described for a diverse range of stresses and in some bacterial species the adaptive response may contribute to pathogenesis (Foster & Hall 1990, Bearson *et al.* 1998, Castanie-Cornet *et al.* 1999). When a stress response is induced, this state may confer a general protection against a range of other stresses, for example a stress response induced by nutrient starvation provides protection against heat stress (Jouper-Jaan *et al.* 1992). *E. faecalis* has been shown to synthesize a variety of stress proteins when exposed to adverse environmental conditions (Hartke *et al.* 1998) including high salt concentration (Flahaut *et al.* 1996a), bile salts, acid, heat (Flahaut *et al.* 1996b), glucose starvation (Giard *et al.* 1997), high pH (Flahaut *et al.* 1997), sodium hypochlorite (Laplace *et al.* 1997) and starvation in tap water (Hartke *et al.* 1998).

If starvation or exposure to sodium hypochlorite solution or diffusing calcium hydroxide induces *E. faecalis* to enter a stress response then this might confer cross protection to *E. faecalis* when it is subsequently exposed to a calcium hydroxide dressing. This study sought to clarify the strategies employed by *E. faecalis* that may result in resistance to treatment. The aim was to investigate the stress response of *E. faecalis* to antimicrobial agents by exposing cells to sublethal concentrations of sodium hypochlorite and calcium hydroxide with or without a range of pretreatments. We also examined alternative cell mechanisms that may be important for survival of *E. faecalis* at high pH.

Materials and methods

Bacterial strain and culture conditions

The bacterial strain used for all experiments was *E. faecalis* JH2-2, which is derived from the parental strain JH2 (Jacob & Hobbs 1974) and is representative of the species. Cultures were grown overnight in 10 mL Brain Heart Infusion broth (BHI, Oxoid Ltd, Basingstoke, UK) at 37°C, in air without shaking. A 2% inoculum in 50 mL BHI was then grown at 37°C to an optical density at 600 nm (OD_{600}) of 1.1. Preliminary experiments showed that cells stopped growing and entered stationary phase at this OD. All experiments described below were repeated in triplicate.

Antimicrobial solutions

Sodium hypochlorite solutions were prepared fresh daily by dilution of a 1% sodium hypochlorite solution (10 000 p.p.m. available chlorine, pH11; Milton®, Procter & Gamble, Parramatta, NSW, Australia) to 0.0001% or 0.005% solution (1 and 50 p.p.m. available chlorine, respectively). Available chlorine was confirmed by colorimetry (Lovibond 1000 Comparitor, Salisbury, UK).

A saturated solution of calcium hydroxide was prepared by dissolving calcium hydroxide powder (Sigma Chemical Co., St Louis, MO, USA) overnight in de-ionized water. The saturated solution was centrifuged (3000 × ***g***, 10 min), the supernatant was removed and filtered using a Millex®-GP 0.22 μm Filter Unit (Millipore, Bedford, MA, USA) resulting in a stock calcium hydroxide solution, pH 11.5. Additional calcium hydroxide solutions of pH 11.1 and 10.3 were prepared by dilution of stock with de-ionized water (1 in 4 and 1 in 40, respectively).

Pre-treatment and challenge conditions

After cultures were grown to stationary phase, 10 mL aliquots were removed and cells were harvested by centrifugation (3000 × ***g***, 10 min). Cells were pretreated by resuspending cell pellets in 10 mL solutions of either 0.0001% sodium hypochlorite solution or calcium hydroxide solution pH 10.3 (or de-ionized water for controls) for 30 min. One mL aliquots were placed in Eppendorf tubes and cells recovered by centrifugation (16 000 × ***g***, 3 min) and washed before challenge.

Table 1 Effect of pretreatment on survival of *E. faecalis* JH2-2 challenged with sodium hypochlorite and calcium hydroxide

	Challenge					
	NaOCl, 0.005%		$Ca(OH)_2$, pH 11.1		$Ca(OH)_2$, pH 11.5	
Pre-treatment	15 min	30 min	15 min	30 min	15 min	30 min
No pretreatment	1.2×10^{-4}	4.4×10^{-6}	4.8×10^{-1}	4.1×10^{-1}	2.6×10^{-4}	1.3×10^{-6}
$Ca(OH)_2$, pH 10.3	1.4×10^{-4}	1.6×10^{-5}	1.0×10^{0}	7.5×10^{-1}	5.7×10^{-4}	6.7×10^{-6}
NaOCl, 0.0001%	8.1×10^{-5}	4.3×10^{-6}	2.3×10^{-1}	1.3×10^{-1}	1.8×10^{-4}	1.7×10^{-6}

Data expressed as percentage survival, average of 3 experiments.
Number of cells at 0 min was $2.3 \pm 0.2 \times 10^9$ (= 100%).

Bacteria were challenged by exposure to sodium hypochlorite (0.005%), or calcium hydroxide solution (pH 11.1 or 11.5). At 0, 15 and 30 min, 1 mL samples were removed, cells recovered by centrifugation (16 000 × ***g***, 3 min), washed, and resuspended in 0.9% sodium chloride solution. Viable counts were determined by serial dilution and plating onto BHI agar. The number of colony forming units (CFU) was determined after incubation for 48 h at 37°C in air.

Mechanisms of survival-response to lethal treatment

To check if protein synthesis was necessary for cell survival, some experiments were repeated in the presence of a protein synthesis inhibitor, chloramphenicol (100 μg mL^{-1}), which was added to the pretreatment and challenge solutions in relevant experiments. Chloramphenicol at a concentration of 100 μg mL^{-1} has been shown to block protein synthesis (Giard *et al.* 1996, Giard *et al.* 1997).

To determine if a proton pump was involved in resistance to high pH, nonadapted cell pellets were exposed to 1 mL calcium hydroxide (pH 11.1) in the presence of a proton pump inhibitor, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP, 100 μM) (ICN Biomedicals Inc., Aurora, OH, USA), for 15 and 30 min. CCCP has been shown to block the normal function of the proton pump at this concentration (Mugikura *et al.* 1990). Control experiments were performed under identical conditions, but with the calcium hydroxide solution adjusted to pH 7.0 by dilution. Cells were recovered and viability determined as above.

Results

Response to sodium hypochlorite

Preliminary experiments revealed that sodium hypochlorite solution at a 0.5 and 1% (v/v) concentration was rapidly lethal, but *E. faecalis* survived a 0.0001% sodium hypochlorite solution (data not shown). Therefore, a sublethal concentration of 0.005% (v/v) sodium hypochlorite was used, which resulted in just 1.2×10^{-4}% of cells surviving after 15 min and 4.4×10^{-6}% cell survival after 30 min exposure (Table 1). Pre-treatment with 0.0001% sodium hypochlorite or calcium hydroxide pH 10.3 for 30 min did not induce tolerance or increase survival of *E. faecalis* in 0.005% sodium hypochlorite (Table 1).

Response to calcium hydroxide

When cells were exposed to calcium hydroxide pH 11.1 for 30 min, 0.4% cells survived, but a relatively small increase in alkalinity to pH 11.5 resulted in a dramatic drop in survival to just 1.3×10^{-6}% (Table 1). Pre-treatment with 0.0001% sodium hypochlorite had no effect on survival of cells challenged with calcium hydroxide pH 11.1 or 11.5 (Table 1). Pre-treatment with calcium hydroxide pH 10.3 for 30 min conferred no resistance by *E. faecalis* to further exposure to calcium hydroxide.

Effect of blocking protein synthesis

The protein synthesis inhibitor, chloramphenicol, was used to block protein production during antimicrobial challenge to determine the role of stress-induced proteins in cell survival. In the presence of 100 μg mL^{-1} chloramphenicol, there was no discernible difference in cell survival when cells were exposed to 0.005% sodium hypochlorite, with or without calcium hydroxide pH 10.3 pretreatment (Table 2). Similarly, there was no difference in cell survival when cells were exposed to calcium hydroxide (pH 11.1 and 11.5) in the presence of chloramphenicol, whether or not they were pretreated with calcium hydroxide pH 10.3 (Table 2).

Table 2 Effect of blocking protein synthesis with Chloramphenicol (Cn) on survival of *E. faecalis* JH2-2 when challenged with sodium hypochlorite and calcium hydroxide, with and without pretreatment

	Challenge					
	NaOCl, 0.005%		$Ca(OH)_2$, pH 11.1		$Ca(OH)_2$, pH 11.5	
Pre-treatment	15 min	30 min	15 min	30 min	15 min	30 min
No pretreatment*	1.2×10^{-4}	4.4×10^{-6}	4.8×10^{-1}	4.1×10^{-1}	2.6×10^{-4}	1.3×10^{-6}
No pretreatment + Cn	1.1×10^{-4}	7.8×10^{-6}	4.9×10^{-1}	3.7×10^{-1}	2.6×10^{-4}	1.6×10^{-6}
$Ca(OH)_2$, pH 10.3*	1.4×10^{-4}	1.6×10^{-5}	1.0×10^{0}	7.5×10^{-1}	5.7×10^{-4}	6.7×10^{-6}
$Ca(OH)_2$, pH 10.3 + Cn	1.1×10^{-4}	9.0×10^{-6}	9.7×10^{-1}	7.5×10^{-1}	7.4×10^{-4}	6.8×10^{-6}

Data expressed as percentage survival, average of three experiments.
Number of cells at 0 min was $2.3 \pm 0.2 \times 10^{9}$ (= 100%).
*Data from Table 1 included for comparison.

Effect of blocking proton pump

When the environmental pH rises, bacteria attempt to preserve the intracellular pH by pumping protons across the cytoplasmic membrane to maintain the cytoplasmic pH (Booth 1985, Booth 1999). The proton pump inhibitor, CCCP, was used to determine whether this mechanism might play a role in tolerance of *E. faecalis* to alkaline conditions.

When cells were exposed to calcium hydroxide at pH 11.1 for 30 min, there was a 20-fold reduction in cell survival in the presence of CCCP compared to calcium hydroxide exposure in the absence of the proton pump inhibitor (Fig. 1). The effect of CCCP was apparently time dependent because the effect was even more pronounced after 60 min of calcium hydroxide exposure (one experiment only; without CCCP 6.4×10^{-3}% of cells survived, but in the presence of CCCP, survival was 9.0×10^{-5}%). Control experiments showed that CCCP was not lethal to *E. faecalis* at pH 7.0 (Fig. 1).

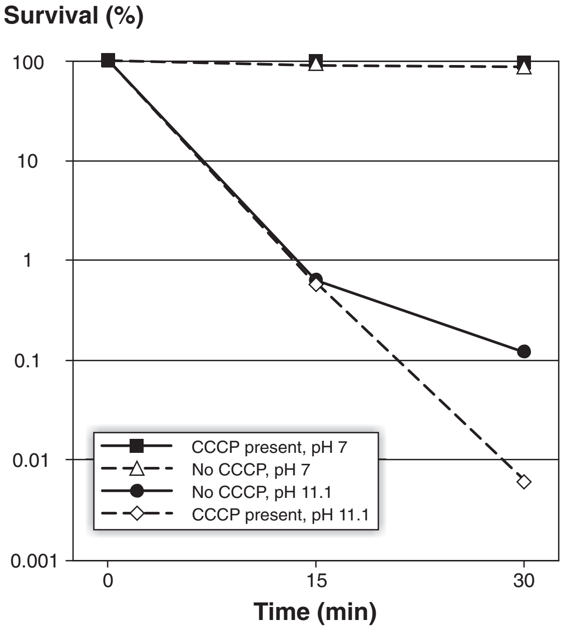

Figure 1 Survival of *E. faecalis* JH2-2 in calcium hydroxide pH 11.1 (and controls, pH 7.0) in the presence or absence of CCCP. Each data point is the average of 3 experiments.

Discussion

The common recovery of *E. faecalis* in root canals of teeth where endodontic treatment has failed implies that this species is intimately involved in the pathogenesis and maintenance of persisting apical periodontitis. One feature that may enable *E. faecalis* to persist in the root canal is an ability for it to survive conventional antimicrobial agents used during endodontic treatment, such as the alkaline pH of calcium hydroxide. *E. faecalis* is known to withstand a high pH; indeed this is an identifying characteristic of *E. faecalis* (Mundt 1986, Devriese *et al.* 1992). At pH 11.5 or greater, *E. faecalis* does not survive, yet it can survive at a pH below 11.5 as shown in this and earlier studies (Byström *et al.* 1985). Because of the buffering effect of dentine (Wang & Hume 1988, Nerwich *et al.* 1993, Haapasalo *et al.* 2000), it is unlikely that the high pH of calcium hydroxide (>11.5) is attained within dentinal tubules where *E. faecalis* has the capacity, at least *in vitro*, to penetrate deeply (Haapasalo & Ørstavik 1987, Ørstavik & Haapasalo 1990, Safavi *et al.* 1990, Vahdaty *et al.* 1993, Peters *et al.* 2000). In radicular dentine, alkalinity may only reach pH 10.3 after dressing the canal with calcium hydroxide (Nerwich *et al.* 1993, Miñana *et al.* 2001).

Because endodontic treatment involves repeated or alternate use of sodium hypochlorite solution and calcium hydroxide at various stages of treatment, *E. faecalis* is likely to be exposed to an alkaline pH, which is sublethal. These conditions could adapt or 'pretreat' cells by eliciting a stress response that enhances their survival. For example, adaptation of *E. faecalis* by incubation at low temperatures confers an increased ability for cells to withstand freezing (Thammavongs *et al.* 1996). It therefore seems reasonable to suspect that repeated exposure of *E. faecalis* to sodium hypochlorite solution and calcium hydroxide might induce resistance to subsequent exposure at levels that might normally be lethal.

The initial experiments confirmed the survival of *E. faecalis* in an alkaline high pH. After 30 min exposure in calcium hydroxide pH 11.1, some cells (0.4%) survived, however, at pH 11.5 >99.99% were killed. Next, *E. faecalis* was pretreated with calcium hydroxide pH 10.3 before challenging cells in calcium hydroxide (pH 11.1 and 11.5) and these experiments revealed a minimal increase in survival by *E. faecalis*, much less than reported in another study (Flahaut *et al.* 1997). Pre-treatment with sodium hypochlorite solution had no effect on survival by *E. faecalis* in calcium hydroxide. Thus, these results show that pretreatment by calcium hydroxide and sodium hypochlorite solution is of minor importance in the ability of *E. faecalis* to survive the high alkalinity of calcium hydroxide.

The capacity to mount a stress response with synthesis of general or specific stress-induced proteins is an important survival mechanism (Foster & Spector 1995). The production of these proteins is a fundamental biological response in many bacterial species and protects cells from adverse conditions, facilitating cell recovery when more favourable conditions are re-established (Parsell & Lindquist 1993, Welch 1993). *E. faecalis* has been shown to produce a number of such proteins in response to other environmental stresses, including glucose starvation (Giard *et al.* 1997), elevated temperatures (Boutibonnes *et al.* 1993) and bile salts (Flahaut *et al.* 1996b). To determine the role of stress-induced proteins in surviving the alkaline pH of calcium hydroxide, protein production was blocked with chloramphenicol during pretreatment and challenge with calcium hydroxide. Stopping protein synthesis had no effect on cell survival, which implies that stress-induced protein production is not important for survival of *E. faecalis* at high pH, a finding consistent with Flahaut *et al.* (1997). The induction of tolerance to high pH was independent of protein synthesis because pretreatment in the presence of chloramphenicol did not alter cell survival.

In acid or alkaline environments, most bacterial cells maintain pH homeostasis in which the internal pH is kept within a narrow range so that enzymes and proteins maintain normal function (Booth 1985). The pH homeostasis is based on two principal components: a passive function consisting of a low cell membrane permeability to ions and a buffering ability of the cytoplasm; and an active mechanism that functions mainly through controlled transport of cations (potassium, sodium and protons) across the cell membrane (Booth 1985, Kakinuma 1998, Booth 1999). In acidic environments, a cation antiport system is thought to raise the internal pH by expelling protons across the cell wall. In an alkaline medium, cations/protons are pumped into the cell to lower the internal pH (Booth 1985, Hall *et al.* 1995, Booth 1999). Extensive work with *Enterococcus hirae* (formerly *Streptococcus faecalis*) has confirmed a fundamental role for a potassium/proton antiport system in maintaining cytoplasmic pH in an alkaline environment (Kakinuma 1987, Kakinuma & Igarashi 1990, Kakinuma & Igarashi 1995, Kakinuma & Igarashi 1999).

In order to determine the role of the proton pump in maintaining survival of *E. faecalis* in a high pH environment, CCCP was used to shut down the pump. CCCP is known to block proton movement across cell membranes (Kinoshita *et al.* 1984). In the presence of CCCP, there was a 20-fold reduction in cell survival after 30 min exposure to high pH compared to cells that were not exposed to CCCP. The effect of CCCP was apparently time-dependent because the effect was more pronounced – a 70-fold reduction – after 60 min of calcium hydroxide exposure (one experiment only). These results show that a functioning proton pump, which drives protons into the cell to acidify the cytoplasm, is critical for the survival of *E. faecalis* in a highly alkaline environment. Presumably, when the environmental alkalinity reaches pH 11.5 or above, this life-saving mechanism is overwhelmed.

During clinical endodontic treatment, sodium hypochlorite solution is usually used in a concentration range of 0.5–5% and preliminary experiments confirmed that exposure to 0.5 and 1% sodium hypochlorite solution for 5 min was lethal to *E. faecalis* (data not shown). Indeed, exposure to 0.005% sodium hypochlorite resulted in >99.99% killing of *E. faecalis* at 30 min. It should be noted that these concentrations are considerably below those used in conventional clinical treatment, yet even at this low concentration sodium hypochlorite solution was highly bactericidal. Pre-treatment with 0.0001% sodium hypochlorite did not induce tolerance against further exposure to sodium hypochlorite, which is consistent with another study (Laplace *et al.* 1997) showing that pretreatment of *E. faecalis* with sodium hypochlorite had no effect on cell survival to further sodium hypochlorite challenge. In all probability, any stress or adaptive response mounted by *E. faecalis* is completely overwhelmed by the lethal effect of sodium hypochlorite.

Several *in vitro* studies have shown sodium hypochlorite solution to be lethal to *E. faecalis*, yet when *E. faecalis*-infected dentine is exposed to sodium hypochlorite solution, some cells of *E. faecalis* may survive (Ørstavik & Haapasalo 1990, Vahdaty *et al.* 1993, Siqueira *et al.* 1997, Heling & Chandler 1998). The recovery of *E. faecalis* in the root canals of failed cases after endodontic retreatment (Sundqvist *et al.* 1998) also implies an ability of *E. faecalis* to survive, albeit rarely, the antimicrobial action of sodium hypochlorite in the root canal. Our findings show that an adaptive stress response to sodium hypochlorite solution does not account for survival of *E. faecalis*. Another possible reason could be strain variation – some strains of *E. faecalis* have been reported to survive low concentrations of sodium hypochlorite (Kearns *et al.* 1995). However, more likely explanations include the possibility that sodium hypochlorite solution occasionally may not reach places where *E. faecalis* may hide, such as in the dentinal tubules or the more inaccessible areas of the root canal system; or, that sodium hypochlorite is buffered by dentine (Haapasalo *et al.* 2000).

In summary, this study has confirmed that *E. faecalis* is resistant to killing by calcium hydroxide at or below pH 11.1. An adaptive response in alkaline pH and stress-induced protein synthesis appear to play minor roles in cell survival, however, a functioning proton pump with the capacity to acidify the cytoplasm is critical for survival of *E. faecalis* at high pH. *E. faecalis* is efficiently killed by sodium hypochlorite; in fact, the concentrations used during clinical treatment are many orders of magnitude higher than that required for lethal action. These findings contribute to our understanding of the response by *E. faecalis* to these antimicrobial agents, yet eradication of *E. faecalis* from an infected root canal remains a significant clinical challenge for modern endodontic treatment.

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